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Plants accumulate TNT and similar compounds from soil. Their sessile nature requires that plants adapt to environmental changes by biochemical and molecular means. In principle, it is possible to develop a monitoring capability based on expression of any gene that is activated by specific environmental conditions. First year Department of Defense Strategic Environmental Research and Development program funding has enabled us to demonstrate the feasibility of using plants to monitor movement of TNT in the biosphere. We have identified plant genes activated upon exposure to TNT and cloned portions of these genes. Partial gene sequences will allow design of DNA probes that measure TNT-induced gene activity. These will be used to develop sensitive exposure assays that monitor gene expression in plants growing in environments contaminated with explosives.

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**Abstract.** Plants accumulate TNT and similar compounds from soil. Their sessile nature requires that plants adapt to environmental changes by biochemical and molecular means. In principle, it is possible to develop a monitoring capability based on expression of any gene that is activated by specific environmental conditions. In the first year of funding, we have demonstrated the feasibility of using plants to monitor movement of TNT in the biosphere. We have identified plant genes activated upon exposure to TNT and cloned portions of these genes. Partial gene sequences will allow design of DNA probes that measure TNT-induced gene activity. These will be used to develop sensitive assays that monitor gene expression in plants growing in environments possibly contaminated with explosives. By monitoring expression of TNT-induced genes, we can monitor whether the plant is exposed to this compound.

**Introduction.** The manufacture and detonation of conventional explosives for military and other purposes worldwide have been extensive. The Cold War left the United States and many other countries with significant quantities of 2,4,6-trinitrotoluene (TNT) in the form of munitions and propellants. Most of these weapons were never used, and because TNT is not stable for long periods, the munitions in steel bomb casings, rockets and artillery shells must be removed, the casing steam cleaned and filled with new explosives. This process produces significant quantities of solid munitions and contaminated aqueous waste. Storage sites containing this effluent can be highly contaminated, and the migration of waste can exceed the boundaries of these controlled areas into public and private land[1]. The migration of munitions into surrounding streams and soils must therefore be monitored.

Plants readily accumulate TNT and structurally related products from hydroponic solutions and soil[2,3] and hydroponic experiments indicate that the absorption capacity is very high[3]. The absorption rate is concentration dependent and is therefore influenced by soil edaphic factors. Higher plants are a major component of most terrestrial ecosystems. Their sessile nature requires that they adapt and adjust to different environmental stresses and exposure to potentially toxic xenobiotic compounds by biochemical means. Plants have therefore evolved a plethora of biochemicals and enzymes they use to tolerate environmental changes and the stresses associated with these. Biochemical processes are genetically regulated. Therefore it is reasonable to expect that changes in expression of genes responsible for regulating these metabolic processes will occur upon exposure to select environmental contaminants.

Plants often accumulate TNT and similar compounds from soil. Once inside the plant, these compounds are rapidly metabolized. This suggests that a change in the pattern of gene expression might occur upon exposure to TNT. The object of the first year of this study was to determine whether TNT-induced changes in gene expression occur in plants and plant cells exposed to TNT. If such changes are apparent, then we would, in subsequent years, identify those genes whose expression is modified by TNT-exposure and develop DNA probes

that could be used to sensitively monitor their expression -- and therefore, indirectly, the presence of TNT in selected environments.

There are several methods to identify genes that are differentially expressed upon exposure to environmental factors. However, until recently, these methods suffered from a severe lack of sensitivity and did not readily produce gene probes that could be used to monitor changes in gene expression. The arbitrarily primed Polymerase Chain Reaction (AP-PCR) RNA fingerprinting technique described by Welsh, *et al.*, [4] and Liang and Pardee[5] is a combination of reverse transcriptase-PCR and Randomly Amplified Polymorphic DNA techniques. We used these methods to identify, clone and sequence cDNA fragments encoded by TNT-induced mRNA sequences. Sequences were compared to those in a database containing previously published sequences to identify particular genes. Sequence information is also being used to develop gene probes that will sensitively monitor gene activity is plant sentinels growing in soils that might contain contaminating TNT. Detection of their activity will be an indication that TNT is present in the environment and plants are accumulating and metabolizing this material.

**Experimental Methods.** Experiments were conducted using rapidly dividing plant suspension cell cultures. Cultures of Datura innoxia were maintained in the dark at 30°C as 50 mL batch suspension cultures as previously described[6]. Cells were grown in modified Gamborg's 1B5 medium supplemented with vitamins[7], and were diluted four-fold every 48 h. Under these conditions, cell number doubles every 24 h.

A stock solution of TNT was prepared by dissolving 25 mg/mL TNT in 95% (v/v) ethyl alcohol. This was stored in the dark at 4°C until ready for use. Twenty-four hours following transfer of cells to fresh media, TNT was added to cell cultures. To account for any artifacts introduced by exposing cells to ethyl alcohol, the TNT solvent, a control culture was exposed to this compound. Cultures were grown for an additional 2, 8 or 13 h before portions of each culture were collected and RNA was extracted.

Total RNA was isolated from plant cells as described by Chomczynski and Sacchi8. Cell suspension (5 mL) was collected from media by gravity filtration, then frozen in liquid nitrogen. Frozen cells were ground to a fine powder with a sterile, liquid nitrogen-cooled mortar and pestle. Frozen powder was transferred to sterile tubes and placed in dry ice to allow the nitrogen to slowly evaporate. An equal volume of hot (>80°C) lysis solution (4 M guanidine thiocyanate, 25 mM sodium citrate, pH. 7.0, 0.5% Sarkosyl, 0.1 M 2-mercaptoethanol) was added while vortexing. Cell debris were removed by centrifugation and cleared lysates were stored at 4°C. RNA was purified from the cleared lysate by extraction with water-saturated phenol and chloroform. RNA was precipitated twice with isopropyl alcohol, collected by centrifugation and the pellets were air dried. Pellets were dissolved in sterile water and RNA concentrations and purity were determined by spectrophotometry. Purified RNA was used immediately for first strand cDNA synthesis or stored at -70°C. The poly(A)$^+$ fraction of RNA was isolated from cleared lysates without prior RNA purification. Messenger RNA was isolated using commercially available purification kits (Promega, Inc., Madison, WI). The procedure was that provided with the kit. Purified mRNA was used as template in first strand cDNA synthesis reactions immediately after spectrophotometric quantitation.

Synthesis of first strand cDNA was primed using decadeoxynucleotides of arbitrary sequence (Table 1). Reactions were performed in sterile 96-well microtiter plates (Nunc, Inc., Denmark). Each 25 µl reaction contained 200 ng total RNA (or 50 ng poly(A)+ RNA), 1 µM of a specific decadeoxynucleotide (Table I), 100 µM each of dATP, dCTP, dGTP, and dTTP, 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl2 and 200 units of M-MLV Reverse Transcriptase (Bethesda, Research Laboratories, La Jolla, CA). RNA was heated to 65°C for five min, then chilled on ice before adding the remainder of the reaction components. Reactions were covered to reduce evaporation and heated at 37°C for 1 h. They were then heated for 15 min at 65°C to inactivate the transcriptase. The newly synthesized first strand of cDNA was stored at 4°C.

Second strand synthesis reactions contained 5 µl of the first strand reaction mix, 25 mM Tris-HCl, pH 8.3, 125 mM KCl, 3.75 mM MgCl2, 200 µM each dATP, dCTP, dGTP and dTTP, 1 µM of the decadeoxynucleotide primer (Table 1) used in the first strand synthesis reaction, and 2 units of AmpliTaq DNA Polymerase (Perkin-Elmer Cetus, Norwalk, CT). Reactions were incubated in a Perkin-Elmer Model 9600 thermocycler. Samples were heated to 94°C for 5 min, cooled to 40°C for 5 min then heated at 72°C for 5 min. Following this treatment, reactions were incubated for 40 cycles. Cycle conditions were 95°C for 30 sec, 40°C for 2 min, and 72°C for 30 sec9. Following a final incubation at 72°C for 5 min, samples were stored at 4°C until analyzed by polyacrylamide gel electrophoresis.

Table 1. Sequences of decadeoxynucleotides used to prime cDNA synthesis.

| Decanucleotide Name | Sequence | Decanucleotide Name | Sequence |
|---|---|---|---|
| AP-21 | CGAGTATGAG | AP-26 | GGAATGCCAT |
| AP-22 | TTGATACGAC | AP-27 | TCCATATCGA |
| AP-23 | GCTCATCAGG | AP-28 | CGGAGTCTGT |
| AP-24 | CTCCTGTAGA | AP-29 | GAAGCTGAGG |
| AP-25 | GATTATCGCC | AP-30 | GAGCACGACT |

Amplified DNA was analyzed by electrophoresis through a 6% nondenaturing polyacrylamide gel (Bio-Rad, La Jolla, CA) to resolve DNA fragments ranging from 0.2 to 1.0 kbp in length. DNA fragments were detected by silver staining[10]. After fixing in 10% (v/v) glacial acetic acid, gels were rinsed in distilled water, then soaked for 20 min in a solution containing 5.9 mM AgNO3, 0.06% (v/v) formaldehyde. Gels were then washed briefly in a solution containing 1.25 mM Na2S2O3, then soaked in a solution containing 283 mM Na2CO3, 0.06% (v/v) formaldehyde, and 1.25 mM Na2S2O3 to precipitate the silver. When the desired level of development was achieved, the reaction was stopped by immersing the gel in a solution containing 10% (v/v) glacial acetic acid for five min. Stained gels were preserved by drying at 80°C under vacuum for 2 h.

DNA fragments that increased in intensity upon exposure of the source cells to TNT were excised from the gel, and placed into 100 µl of PCR reaction mix [10 mM Tris-HCl, pH 8.3, 10 mM KCl, 4 mM MgCl2, 200 µM each dATP, dCTP,

dGTP and dTTP, 1 μM of the decadeoxynucleotide used in the original amplification reaction, and 10 units of AmpliTaq DNA polymerase Stoffel fragment (Perkin-Elmer Cetus, Nowalk, CT)]. DNA was re-amplified from the dried gel with no further purification by 40 cycles under the following conditions: 94°C for 30 sec, 40°C for 2 min, and 72°C for 30 sec. After a final incubation at 72°C for 5 min, reactions were stored at 4°C. Polyacrylamide gel electrophoresis of the reaction products showed DNA fragments the same size as those used as template in the re-amplification reaction.

Amplified DNA fragments were cloned and sequenced. PCR-amplified DNA fragments were ligated to the plasmid vector pGEM-T (Promega, Inc, Madison, WI). Ten μl of the above PCR amplification reaction was mixed with 5.3 ng pGEM-T DNA and 1 Weiss unit of T4 DNA Ligase (Promega, Inc.) in 15 μl of a solution containing 30 mM Tris-HCl, pH 7.6, 10 mM MgCl2, 10 mM dithiothreitol and 0.5 mM ATP. The entire 15 μl ligation reaction was used to transform competent DH5α *E. coli* cells. Resulting transformants were plated on LB agar plates containing 50 μg/ml ampicillin, 40 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and 200 μg/ml isopropyl-β-D-thiogalactopyranoside. After overnight incubation at 37°C, well-separated colorless colonies were picked to fresh plates and incubated at 37°C as before. Fresh colonies were used to inoculate liquid cultures. Plasmid DNA was extracted and purified from cultures and was sequenced by the Sanger dideoxyl method using dye-terminators (Applied Biosystems). DNA sequences were determined on an Applied Biosystems Model 373A automated DNA sequencer. DNA sequences obtained in this manner were analyzed and compared to published sequences using GenBank software developed at Los Alamos.

**Results and Discussion.**

TNT uptake by cell cultures. Growth of cell cultures in media containing different TNT concentrations followed by analysis of the culture media for TNT content establishes the rate of TNT uptake by cultures (Figure 1). After two d cell growth, the TNT concentration was reduced to less than 1 ppm suggesting an active accumulation of TNT by cell cultures. The mechanism of TNT uptake is not understood, but less than 1% was associated with external plant cell walls suggesting uptake across the cell membrane. While TNT is rapidly removed from the media, significant concentrations do not accumulate in the cells. [$^{14}$C]-TNT is only a minor component of the total radiolabeled metabolites present within the cells suggesting that it is modified shortly after entering. Exposure to [$^{14}$C]-TNT, causes accumulation of relatively polar [$^{14}$C]-metabolites. These appear within 30 minutes following TNT exposure. After 24 hours, at least two distinct, broad peaks on reverse phase HPLC columns are detectable (data not shown). Their retention times suggest that these compounds are not dinitrotoluene isomers. The most polar metabolite produced by the cells was analyzed by liquid chromatography-mass spectroscopy (LC-MS). This compound has a molecular weight of 197 and, depending on the ionization conditions, fragments of varying intensity are seen at 181, 169, 151 or 149 m/z. The most likely TNT metabolite compatible with this fragmentation pattern is diaminonitrobenzoic acid. This suggests that at least two of the nitrate groups can be reduced and that the methyl group can be oxidized by enzymes present in

the cells. None of the mass spectral or HPLC data suggested that TNT was conjugated with hexoses.

Growth of cell cultures in different TNT concentrations. D. innoxia cell cultures were grown in media containing different TNT concentrations and

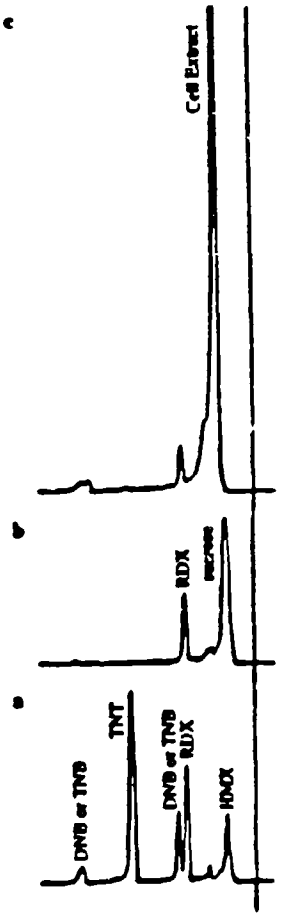


Figure 1. HPLC profiles of solutions containing explosives. a) A solution of "pink water" containing different explosive compounds prior to treatment with *Datura innoxia* cells. b) The same solution after treatment with these cells. c) An extract of *D. innoxia* cells used to remove explosive compounds from the waste solution. The large peak with the shortest retention time (right) in panel b is sucrose from the plant cell media. The large peak with the same retention time in panel c results from sucrose and other organic compounds released from the cells upon extraction. Independent experiments demonstrate removal of HMX from these waste solutions.

cell growth was measured as described previously[7]. While cell cultures rapidly remove TNT from the media at concentrations above 100 ppm, long-term exposure to concentrations above 25 ppm is lethal (Figure 2). A marked decrease in cell division occurs at 20 ppm, but 10 ppm has no significant impact on cell division rates or culture viability. Intact plants can tolerate significantly higher TNT concentrations, probably because they are not directly exposed to solutions containing these high concentrations. TNT concentrations that significantly impact cell division were chosen for gene expression studies.

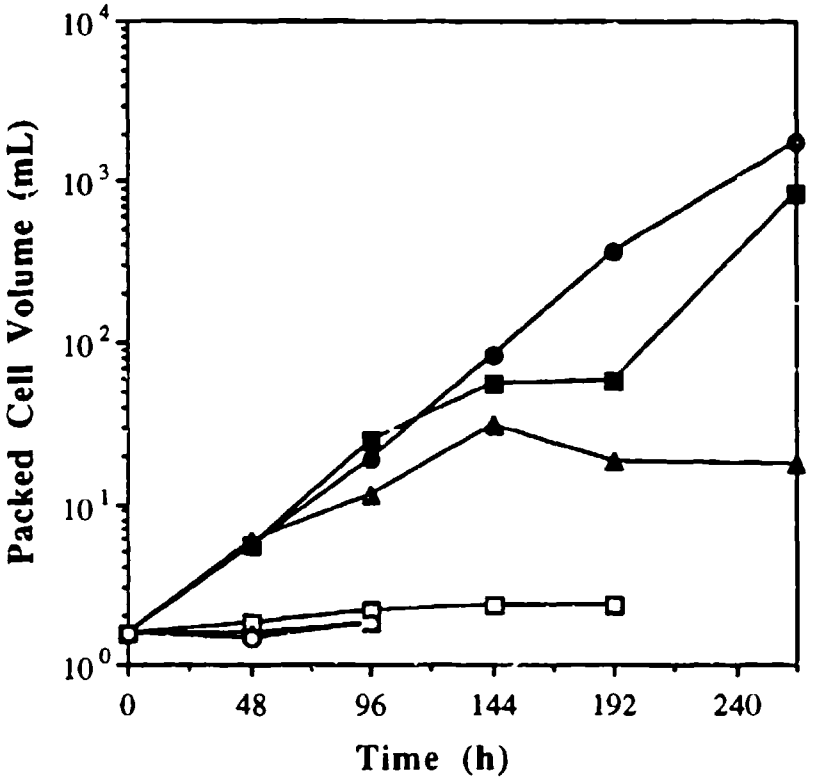


Figure 2. D. innoxia cell cultures were grown in log phase, then transferred to media containing different TNT concentrations. Cell growth, as determined by packed cell volume, was determined at two day intervals after transfer to media containing TNT. Cells were diluted as necessary to maintain cell density in a range optimal for rapid growth. Control cells (no TNT), - • -; cells grown in 10 ppm TNT, - ■ -; cells grown in 20 ppm TNT, - ▲ -; cells grown in 30 ppm TNT, - □ -; cells grown in 40 ppm TNT, - Δ -; and cells grown in 50 ppm TNT, - o -.

Analysis of gene expression. Upon exposure to toxic or deleterious conditions, plants and plant cells change their pattern of gene expression. These changes can be catagorized as rapid, transient changes, and rapid, but long-term responses. The former changes occur as genes are up- or down-regulated immediately upon exposure to a change in their environment. However, within several hours, expression returns to previous levels. The latter responses also result in rapid up- or down-regulation, but the modified expression state remains constant as long as the cells are exposed to the inducing conditions and returns to previous levels only when the causative agent is removed from the environment. Rapid, long-term responses are most important for this study. However, all genes that are up- or down-regulated in D. innoxia cells exposed to TNT are of interest. Many of the apparent changes will probably be typical of those caused by other chemically and environmentally

induced stress. However, a subset of these responses may be specific to TNT-exposure.

Messenger RNA is the initial product of an expressed gene. Therefore the most direct method of monitoring gene expression is to monitor the mRNA encoded by that gene. Analysis of mRNA in the absence of specific gene probes

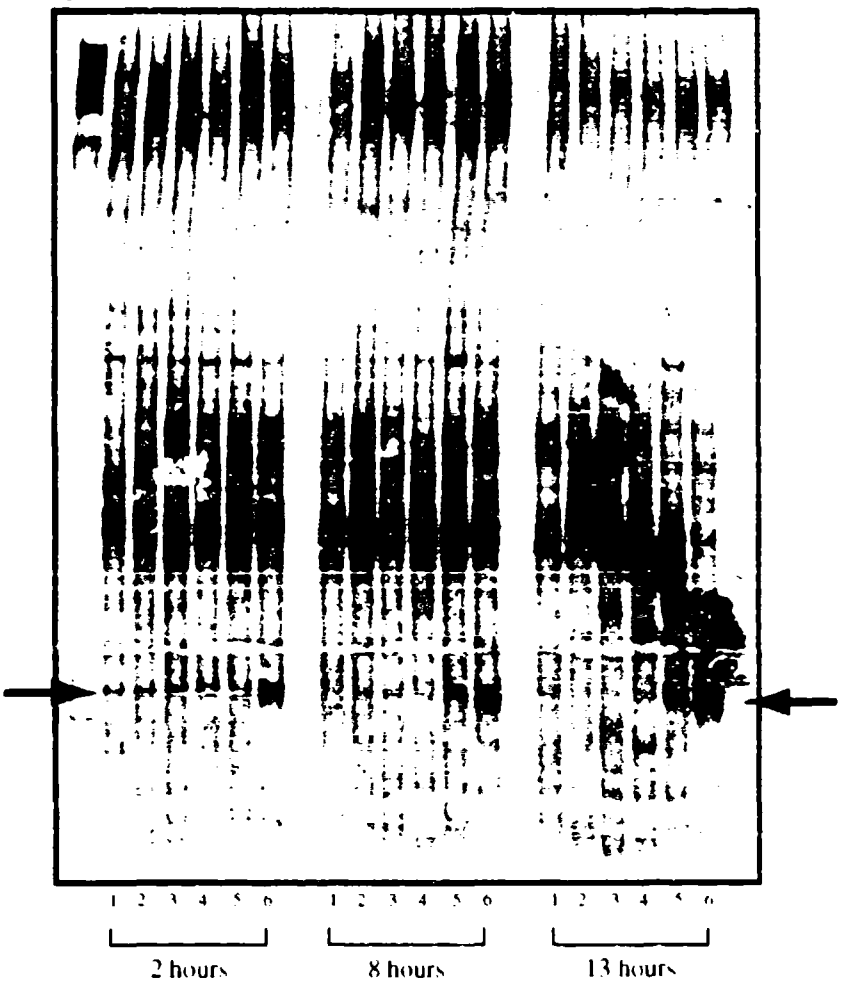


Figure 3. *Datura innoxia* cells were grown in (1) 0 TNT; (2) 1.6 µl/ml added ethanol; (3) 12.5 ppm TNT; (4) 25 ppm TNT; (5) 50 ppm TNT; or (6) 100 ppm TNT for 2, 8 or 13 hours. Total RNA from each sample was used as template in rtPCR reactions primed with a single decadeoxynucleotide (5'-CTCCTGTAGA-3'). A portion of this product was used as template in a PCR reaction primed with the same DNA decamer. Reactions were analyzed by electrophoresis through a 4% non-denaturing polyacrylamide gel and fragments were visualized by silver staining. Arrows indicate a DNA fragment that increases in reactions containing RNA template from cells exposed to higher TNT concentrations for increasing periods. Reactions using other DNA decamers identify other TNT-induced sequences.

has historically been accomplished by using the mRNA to drive *in vitro* translation reactions that produce radiolabeled proteins encoded by the mRNA's present. Products of these reactions are then analyzed by two-dimensional polyacrylamide gel electrophoresis, and the proteins are visualized by autoradiography. While this procedure demonstrates differences in gene

expression, it does not provide a method of identifying and isolating the gene encoding any protein of interest. To overcome this problem, total RNA was isolated at different times following TNT exposure and used as template in reverse transcriptase PCR (rtPCR) reactions primed by single decadeoxynucleotides whose sequences were chosen randomly (Table 1). The resulting single-stranded cDNAs were used as templates in further PCR reactions to generate a series of short DNA fragments. Since the production of these fragments is dependent upon the constitution of the original mRNA template population, changes in the frequency of selected mRNA's are manifested as changing populations of PCR products. These DNA fragments were analyzed on non-denaturing polyacrylamide gels, then stained to visualize the DNA. Figure 3 shows a typical result. RNA was extracted from cells growing in different TNT concentrations for 2, 8 and 13 h and used as template in rtPCR, then PCR reactions containing a single DNA decamer primer. Concentrations of a particular DNA fragment (arrows) clearly change upon exposure to increasing TNT for longer times.

**Figure 4.** DNA Sequences of PCR Amplicons amplified from TNT-induced mRNA†.

**Clone AP21.1**

001 CGAGTATGAGCCCAGATCTAGGGGGACTTTAACAGCAACGTGTAAACAAATATGGGAACAT
061 TCAAGAAAAGGAAATGCTCATAACCCAGGGAGATCAGAAACATAT TACACAACATAT NAT
121 AAAA TGTGT T TCTTCAGT TCTCT TCAT TCACATCATACTCCTCATACTCGAATCACTAGT
181 GCGGCCGCCGGCAGGTCGACCATATGGGAGAGCTCCCAACGCGT TGGATGCATAGCTTGA
241 GTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTT

**Clone AP29.1**

001 GAAGCTGAGGCCAA AGAAGAAGATCAAGTAGGCATGACATCCT T TCTAGTAGATCAATTG
061 TGTGAATTGC/.GT TATACTGCCAGTCTGTGGTCT TACAGT TT TCTGT T TNGNGTTCAAAT
121 TGCTGTAGTGGATAGACTAGTAGT TGTCT TAAAA CGTCAATAT TATCGGATAAGTGCAAG
181 CT TACACTAGCGTGACT TAT TCTAATATCCAT T TGTGAACCCTCAGCTTCAATCACTAGT
241 GCGGNCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATG

†PCR amplicons produced only in reactions containing template RNA from TNT-exposed *D. innoxia* cells was cloned into the plasmid vector pGEM-T and the DNA insert was sequenced. Clone AP21.1 has no homology with any published sequences while, clone AP29.1 has a sequence very similar to the tobacco ubiquitin gene.

Use of different randomly selected decadeoxynucleotides to prime the rtPCR and PCR reactions results in amplification of different mRNA sequences. Reactions containing the different DNA decamers shown in Table 1 plus mRNA template from cells growing for different times in differing TNT concentrations amplified seven different TNT-induced fragments. These were cut from the polyacrylamide gels, reamplified and cloned into a plasmid vector. Cloned DNA fragments were then sequenced. Sequences of two such clones are shown in Figure 4. These DNA sequences were compared to all published sequences present in the GenBank DNA database. Clone AP21.1 has no apparent sequence homology with any published sequence and probably encodes an uncharacterized gene. Clone AP29.1 has 71% sequence homology with the 3' UTR of tobacco ubiquitin. Ubiquitin functions in the degradation of proteins and

it is well established that the gene encoding this protein is induced in plants by heat shock, exposure to toxic metal ions and other deleterious environmental factors. It is therefore not surprising that it is induced by high TNT concentrations. While isolation of a *D. innoxia* ubiquitin gene does not provide a specific TNT-induced gene probe, it demonstrates that the methods used do identify TNT-induced genes in plants.

## CONCLUSIONS

The experiments described here demonstrate that external TNT concentrations cause changes in plant gene expression that can be detected using molecular techniques. Such results strongly support the feasibility of using methods to monitor these changes in gene expressions as a means to indirectly monitor changing TNT concentrations in soils and waste streams. PCR amplification using ten different decadeoxynucleotides as primers in reactions containing RNA template from TNT-treated cells produced seven different unique PCR amplicons. While different primers primed three different portions of a single, TNT-induced mRNA sequence, the remaining four primers produced products that encode genes that were previously unknown. These results suggest that this method will identify genes that are specifically induced by TNT and provide a method to develop molecular probes to monitor their activity. The gene identified by three different primers encodes the *D. innoxia* ubiquitin gene. Ubiquitin binds to damaged proteins facilitating their degradation. Heat shock, exposure to toxic metal ions and many other environmental conditions detrimental to plants induce ubiquitin genes. Therefore it is not surprising that ubiquitin gene expression increases upon exposure to high TNT concentrations since TNT reduces culture viability significantly. Identification of these ubiquitin-specific gene probes using the methods described here demonstrates that this approach functions to identify TNT-induced genes. All the TNT-induced DNA fragments amplified by this process are being sequenced and will be used to design and construct DNA oligomers for use in PCR-based detection methods to monitor expression of the genes they represent. The remaining 80 RAPD primers will be used to identify additional TNT-induced genes. The results obtained in the first year demonstrated the feasibility of growing plants in soils suspected of containing environmental pollutants, then monitoring their gene expression to determine whether TNT is present in their environment.

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